(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 January 2001 (18.01.2001)

PCT

(10) International Publication Number
WO 01/04313 A1

(51) International Patent Classification[7]: C12N 15/24, 15/63, 15/67, 15/85, C07K 14/54, A61K 31/713

(21) International Application Number: PCT/US00/17670

(22) International Filing Date: 27 June 2000 (27.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/143,059 9 July 1999 (09.07.1999) US

(71) Applicant *(for all designated States except US)*: AMERICAN HOME PRODUCTS CORPORATION [US/US]; Five Giralda Farms, Madison, NJ 07940 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SATISHCHANDRAN, C. [US/US]; 605 Shepard Drive, Lansdale, PA 19446 (US). PACHUK, Catherine, J. [US/US]; 3212 New Park Circle, Lansdale, PA 19446 (US).

(74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW). Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/04313 A1

(54) Title: METHODS AND COMPOSITIONS FOR PREVENTING THE FORMATION OF ABERRANT RNA DURING TRANSCRIPTION OF A PLASMID SEQUENCE

(57) Abstract: Polynucleotide molecules, which include single stranded DNA or RNA, partially double-stranded DNA, and double-stranded DNA molecules, contain terminator sequences and/or other modifications which suppress the production of unwanted polynucleotide species from these molecules when transfected in a host cell. These molecules are useful in methods for enhancing the efficiency of transcription of a selected polynucleotide sequence in a transfected host cell, and reducing the potential for the products of unwanted transcripts. Further, the methods of the invention are useful in avoiding extinguishing or down regulating the expression of certain polynucleotides present in a host cell or host. These compositions and methods are useful in therapeutic, vaccine, diagnostic and research fields.

METHODS AND COMPOSITIONS FOR PREVENTING THE FORMATION OF ABERRANT RNA DURING TRANSCRIPTION OF A PLASMID SEQUENCE

Field of the Invention

The present invention relates to methods and polynucleotide compositions useful for enhancing the efficiency of expression of polynucleic acid sequences in a host cell by decreasing the expression of undesirable polynucleic acid sequences. More specifically, the invention relates to novel compositions and methods for preventing the expression of aberrant DNA and RNA sequences by use of such compositions.

Background of the Invention

Polynucleotide compositions have been described for pharmaceutical uses, primarily for treatment or prophylaxis of disease in mammals, as well as in research in such fields. Specifically a great deal of activity presently surrounds the use of polynucleotide compositions in the treatment of pathogenic extracellular and intracellular infections, such as viral, bacterial, fungal infections, and the like. As one example, DNA vaccines are described to deliver to a mammalian cell in vivo an agent which will combat a pathogen by harnessing the mammalian immune system. Thus, such vaccines are designed to express, for example, a viral protein or polypeptide, and elicit a humoral or cellular immune response upon challenge by the infective agent. Gene therapy vectors, on the other hand, are polynucleotide compositions generally designed to deliver to a mammalian cell a protein which is either not expressed, expressed improperly or under expressed in a mammal. Such vectors frequently must address species specific immune responses to the those polynucleotide sequences that are recognized as antigenic or which evoke an unwanted cellular immune response. Still other therapeutic uses of polynucleotide compositions are for the delivery of missing or under expressed proteins to a diseased mammalian patient. Furthermore,

polynucleotides are useful themselves as *in vivo* reagents, in diagnostic/imaging protocols, as reagents in gene therapy, in antisense protocols, in vaccine applications or otherwise as pharmaceuticals used to treat or prevent a variety of ailments such as genetic defects, infectious diseases, cancer, and autoimmune diseases. Polynucleotides are also useful as *in vitro* reagents in assays such as biological research assays, medical, diagnostic, screening and contamination detection assays.

A host of problems well-known to the art has prevented the numerous polynucleotide compositions from becoming widely accepted as useful pharmaceutics. Thus, there are few such DNA vaccines or therapeutics which have yet been accepted by the medical community for the treatment of disease in mammals.

Phenomena have been observed in plants, nematodes, and Drosophila that are mediated by polynucleotide compositions, and are referred to as post-transcriptional gene silencing and transcriptional silencing. This phenomenon demonstrates that transfection or infection of a plant, nematode or Drosophila with a virus, viroid, plasmid or RNA expressing a polynucleotide sequence having some homology to a regulatory element, such as a promoter or a native gene or a portion thereof already expressed in that cell, can result in the permanent inhibition of expression of both the endogenous regulatory element and/or gene and the exogenous sequence. This silencing effect was shown to be gene specific. See, for example, L. Timmons and A. Fire, Nature, 395:354 (Oct. 29, 1998); A. Fire *et al*, Nature, 391:806-810 (Feb. 19, 1998); R. Jorgensen *et al*, Science, 279:1486-1487 (March 6, 1998); J. R. Kennerdell and r. W. Carthew, Cell, 95:1017-1026 (Dec. 1998); L. Misquitta and B. M. Paterson, Proc. Natl. Acad. Sci., USA, 96:1451-1456 (Feb. 1999); M. K. Montgomery et al, Proc. Natl. Acad. Sci., USA, 95:15502-15507 (Dec. 1998)]. A DNA plasmid encoding a full-length pro-alpha 1 collagen gene was transiently transfected into a rodent fibroblast tissue cell line and a "silencing" effect on the native collagen gene and the transiently expressed gene observed [Bahramian and Zarbl, Mol. Cell. Biol., 19(1):274-283 (Jan. 1999)].

Still another issue with the use of polynucleotide molecules is the formation of aberrant RNA or DNA, instead of the gene-containing transcript intended. The

formation of aberrant RNA or DNA has been postulated to occur in the use of any transfected plasmid or polynucleic acid molecule and to reduce the efficiency of the target gene expression. The formation of aberrant or abnormal RNA can be one cause of the silencing effect noted above.

There are available and known in the art many sequences which function to terminate transcription. For example, a pause site for RNA polymerase II is associated with termination of transcription, particularly when the site is positioned immediately downstream of a strong polyadenylation signal in a transient expression system [P. Enriquez-Harris *et al*, EMBO J., 9(7):1833-1842 (1991); G. W. Hatfield *et al*, Mol. Cell. Biol., 3(10):1687-1693 (1983)].

However, to date, no suggestion has been made to create an effective and functional composition or method to harness termination mechanisms for use in increasing the efficiency of polynucleotide expression in the pharmaceutical, vaccine, gene therapy and diagnostic fields. There exists a need in the art for polynucleotide compositions and methods of using same to inhibit the formation of aberrant RNA or DNA molecules and increase expression of a selected polynucleotide sequence in a host cell.

Summary of the Invention

In one aspect, the invention provides a double stranded polynucleotide (preferably deoxyribonucleic acid) molecule comprising a first coding strand and a second transcription template strand. The first strand comprises (i) at least one expression cassette sequence which comprises, from 5' to 3', a promoter, a selected polynucleotide sequence the expression of which is controlled by the promoter, and a polyadenylation site, and (ii) at least one first strand terminator sequence. The selected polynucleotide sequence of the expression cassette can be any polynucleotide sequence which is desired to be expressed in a cell to perform a biological function. The first strand terminator sequence is preferably located 5' to the promoter, located 3' to the polyadenylation site, located on the first strand outside of the expression cassette sequence, or located within the selected polynucleotide sequence of the expression

cassette sequence. The first strand termination is located in a position which does not impede transcription from the sequence on the second strand which is complementary to the expression cassette sequence, nor effect the function of the polynucleotide sequence to be expressed. The second strand is complementary to the first strand. The portions of the second strand sequence complementary to the first strand terminator sequence do not impede transcription from the second strand sequence complementary to the first strand expression cassette. Further, the second strand comprises at least one second strand terminator sequence which terminates transcription initiated on the second strand. The second strand terminator sequence is preferably located on the second strand outside of the sequence complementary to the expression cassette of the first strand and in a position which does not impede transcription from the sequence complementary to the expression cassette sequence, nor impairs the biological function of the polynucleotide sequence, when expressed.

In another aspect, the invention provides a pharmaceutical composition comprising the above-identified double-stranded polynucleotide molecule, an optional agent that facilitates polynucleotide (e.g., DNA) uptake in a cell, and a suitable pharmaceutically acceptable carrier. Such compositions are useful for treating intracellular pathogenic infections, such as viruses. Other such compositions are useful for treating certain cancers. Other such compositions are useful for treating certain extracellular pathogens.

In still another aspect, the invention provides a single stranded polynucleotide sequence selected from a first strand or second strand of a double stranded polynucleotide molecule described above.

In yet another aspect, the invention provides a pharmaceutical composition comprising the above-identified single-stranded polynucleotide molecule, an optional agent that facilitates polynucleotide (e.g., DNA) uptake in a cell, and a suitable pharmaceutically acceptable carrier.

In still another aspect, the invention provides a method for enhancing the efficiency of expression of a selected polynucleotide sequence in a host cell, the method comprising the step of transfecting the host cell with a double stranded DNA

molecule described above or a single strand thereof, and thereby inhibiting the formation of aberrant polynucleotide sequences transcribed from the polynucleotide molecule in the host cell.

In another aspect the invention provides a single stranded RNA molecule comprising a sequence of ribonucleic acids having a 5' end and a 3' end, modified to prevent the formation of double-stranded or partially double-stranded regions. In one embodiment, this molecule comprises a 5' cap. In another embodiment, this molecule does not have a cap. In another embodiment, this molecule has a 3' polyA tail. In still another embodiment, this molecule has no polyA tail.

In still a further aspect, the invention provides a pharmaceutical composition comprising the above-identified single-stranded RNA molecule, an optional agent that facilitates RNA uptake in a cell, and a suitable pharmaceutically acceptable carrier.

In another aspect, the invention provides a method for enhancing the efficiency of expression of a selected polynucleotide sequence in a host cell, the method comprising the step of transfecting the host cell with the single-stranded RNA molecule described above, and thereby inhibiting the formation of aberrant RNA molecules in the host cell.

In yet a further aspect, the invention provides a method for treating a mammalian subject comprising administering an effective amount of a pharmaceutical composition comprising any of the polynucleotide molecules described above, an optional agent that facilitates DNA or RNA uptake in a cell, and a suitable pharmaceutically acceptable carrier.

In yet another aspect, the invention provides a method for preventing the unintentional shutting off or down-regulation of a polynucleotide sequence in a host cell transfected with a polynucleotide molecule containing a selected polynucleotide sequence homologous to the polynucleotide sequence present in the host cell, the method comprising the steps of : administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule as described above, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier.

In one embodiment, the polynucleotide compositions or molecules are made by enzymatic synthetic methods or chemical synthetic methods *in vitro*. In another embodiment, the compositions or molecules may be generated in a recombinant culture, e.g., bacterial cells, isolated therefrom, and used in the methods discussed below. In another embodiment the composition generates the polynucleotide molecule *in vivo* after delivery to the host cell.

Still another aspect of the present invention provides such compositions and molecules for use in research methods, such as a reagent for reducing or inhibiting undesired polynucleotide expression in host cells or tissue *in vitro* for use in diagnostic or other research assays, or *ex vivo* for return to the host subject for therapy or other medicinal uses.

Other aspects of the invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Figures

Fig. 1A is an illustration of a double-stranded DNA molecule of this invention containing a first "sense" strand, with the 5' terminator sequence (T^1), the promoter (P), the selected polynucleotide sequence (PN), the polyadenylation sequence (pA), the 3' terminator sequence (T^2) and containing a second complementary strand containing the second strand terminator sequence (T^3), which in this case, is located in a position which is 3' relative to the polyA sequence on the sense strand. Either the first or second strand may be utilized as a single-stranded DNA molecule of this invention.

Fig. 1B is an illustration of another double-stranded DNA molecule of this invention containing a first "sense" strand, with two 5' terminator sequences (T^1 and T^2), the promoter (P), the selected polynucleotide sequence (PN), the polyadenylation sequence (pA), the 3' terminator sequence (T^3) and an RNA instability sequence (RIS); and containing a second complementary strand containing multiple second strand terminator sequences (T^4 through T^8) interspersed in the region which is not complementary to the expression cassette region on the first strand. In this case, terminators T^4 and T^5 are located in positions downstream relative to the polyA

of the DNA encoding the subunits and the IRES is less than five kilobases. However, the selected polynucleotide may encode any product desirable for study. The selection of the selected polynucleotide sequence is not a limitation of this invention.

Other useful products encoded by the selected polynucleotide include hormones, growth and differentiation factors including, without limitation, insulin, glucagon, growth hormone (GH), parathyroid hormone (PTH), growth hormone releasing factor (GRF), follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), vascular endothelial growth factor (VEGF), angiopoietins, angiostatin, granulocyte colony stimulating factor (GCSF), erythropoietin (EPO), connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF), transforming growth factor α (TGFα), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), any one of the transforming growth factor β superfamily, including TGF β, activins, inhibins, or any of the bone morphogenic proteins (BMP) BMPs 1-15, any one of the heregulin/neuregulin/ARIA/neu differentiation factor (NDF) family of growth factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins NT-3 and NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), neurturin, agrin, any one of the family of semaphorins/collapsins, netrin-1 and netrin-2, hepatocyte growth factor (HGF), ephrins, noggin, sonic hedgehog and tyrosine hydroxylase.

Other useful selected polynucleotide products include proteins that regulate the immune system including, without limitation, cytokines and lymphokines such as thrombopoietin (TPO), interleukins (IL) IL-1 through IL-17, monocyte chemoattractant protein, leukemia inhibitory factor, granulocyte-macrophage colony stimulating factor, Fas ligand, tumor necrosis factors α and β, interferons α, β, and γ, stem cell factor, flk-2/flt3 ligand. Gene products produced by the immune system are also useful in the invention. These include, without limitations, immunoglobulins IgG, IgM, IgA, IgD and IgE, chimeric immunoglobulins, humanized antibodies, single chain antibodies, T cell receptors, chimeric T cell receptors, single chain T cell receptors,

class I and class II MHC molecules, as well as engineered immunoglobulins and MHC molecules. Useful gene products also include complement regulatory proteins such as complement regulatory proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF), CR1, CF2 and CD59.

Still other useful selected polynucleotides may produce a product such as any one of the receptors for the hormones, growth factors, cytokines, lymphokines, regulatory proteins and immune system proteins. The selected polynucleotide may be a receptor for cholesterol regulation, including the low density lipoprotein (LDL) receptor, high density lipoprotein (HDL) receptor, the very low density lipoprotein (VLDL) receptor, and the scavenger receptor. The selected polynucleotide sequence useful in this invention also encodes products such as members of the steroid hormone receptor superfamily including glucocorticoid receptors and estrogen receptors, Vitamin D receptors and other nuclear receptors. In addition, useful polynucleotide sequences include sequences encoding transcription factors such as *jun*, *fos*, max, mad, serum response factor (SRF), AP-1, AP2, *myb*, MyoD and myogenin, ETS-box containing proteins, TFE3, E2F, ATF1, ATF2, ATF3, ATF4, ZF5, NFAT, CREB, HNF-4, C/EBP, SP1, CCAAT-box binding proteins, interferon regulation factor (IRF-1), Wilms tumor protein, ETS-binding protein, STAT, GATA-box binding proteins, e.g., GATA-3, and the forkhead family of winged helix proteins.

Other useful products which may be encoded by the selected polynucleotide sequence of the expression cassette include, carbamoyl synthetase I, ornithine transcarbamylase, arginosuccinate synthetase, arginosuccinate lyase, arginase, fumarylacetacetate hydrolase, phenylalanine hydroxylase, alpha-1 antitrypsin, glucose-6-phosphatase, porphobilinogen deaminase, factor VIII, factor IX, cystathione beta-synthase, branched chain ketoacid decarboxylase, albumin, isovaleryl-coA dehydrogenase, propionyl CoA carboxylase, methyl malonyl CoA mutase, glutaryl CoA dehydrogenase, insulin, beta-glucosidase, pyruvate carboxylate, hepatic phosphorylase, phosphorylase kinase, glycine decarboxylase, H-protein, T-protein, a cystic fibrosis transmembrane regulator (CFTR) sequence, and a dystrophin cDNA sequence.

Still other useful polynucleotide-encoded products include, non-naturally occurring polypeptides, such as chimeric or hybrid polypeptides having a non-naturally occurring amino acid sequence containing insertions, deletions or amino acid substitutions, e.g., single-chain engineered immunoglobulins, antisense molecules and catalytic nucleic acids, such as ribozymes. Still other suitable polynucleotide sequences may be readily selected by one of skill in the art. The selection of the polynucleotide is not considered to be a limitation of this invention. As shown in the following examples, an illustrative selected polynucleotide sequence is the sequence encoding murine interleukin-12.

As part of the first strand are at least one, and desirably at least two, and preferably three or more transcriptional terminator sequences. Transcriptional terminators can be located anywhere on the DNA coding (i.e., the non-transcribed) strand, including within the expression cassette sequence, as long as transcription can proceed uninterrupted within the expression cassette on the DNA template ("second") strand and the biological function of the polynucleotide sequence in the expression cassette is not adversely effected upon expression. Essentially, any modification may be made to the first strand to incorporate the transcription terminators, so long as transcription can take place from the one or more expression cassette complementary region(s) of the second "template" strand and the expressed protein or peptide retains its biological function. Preferred transcription terminators do not create secondary structures that interfere with transcription of the template strand. The function of these terminator sequences is to prevent unwanted transcription from occurring from the first strand, and also from the complementary second transcription template strand, when these strands of the DNA molecule are in the host cell. Importantly, these first strand transcription terminators cannot impede or interrupt the necessary transcription from the second template strand or from an expression cassette intended to be transcribed from the first strand of a bicistronic molecule. For example, the function of these terminator sequences prevents the formation of undesired transcripts which, if homologous to a polynucleotide sequence present in the host cell, could shut off or down regulate its normal transcription in the host cell. Further, the function of these

terminators prevents physical occlusion of the promoter, thereby increasing the efficacy of the expression of the selected polynucleotide. Where the first strand is bicistronic (i.e., contains two or more expression cassettes or each strand contains an expression cassette), the same constraints are placed upon the identity and location of the terminator sequence(s).

Depending upon the number of terminator sequences used in an individual first strand, each terminator sequence may be the same or a different terminator sequence. In one embodiment, a terminator sequence may be a bacterial terminator, such as the 5S ribosomal RNA terminator, rrnB. In another embodiment, a terminator sequence may be a bacterial terminator, such as the tryptophan operon terminator, trpA. In another embodiment, a terminator sequence may be a bacteriophage terminator, e.g., a lambda 5S RNA terminator or the bacteriophage P1 head protein terminator. Still another desirable terminator sequence which may be employed positioned 3' to a polyA site is a eukaryotic pause site for RNA pol II, such as the site described in P. Enriquez-Harris *et al*, EMBO J., 10(7):1833-1842 (1991). An α-globin terminator is an example of a terminator for RNA pol II and may also be employed as a terminator sequence in a first strand of this molecule. Further, a histone mRNA processing signal, such as that described in N. Chodchoy *et al*, Mol. Cell. Biol., 11(1):497-509 (Jan. 1991), is a useful terminator sequence for the first strand. Similarly the terminator for the mammalian gastrin gene, the gastrin terminator, is also useful. Another useful terminator sequence is a polynucleic acid sequence that provides a ribozyme cleavage site followed by a pause site terminator sequence, as described above. Other terminator sequences for use in the first strand include a ribonucleic acid cleavage site followed 3' by a pause site terminator sequence. Both rho-dependent terminators [e.g., C. E. Bogden *et al*, Mol. Cell, 3:487-493 (Apr. 1999)] and rho-independent terminators [e.g., I. Gusarov and E. Nudler, Mol. Cell, 3:495-504 (Apr. 1999)] are also sequences which may be employed for this purpose in the first strand.

Additionally, a so-called "padlock probe" described in M. Nilsson *et al*, Science, 265:2085-2088 (1994) is a useful terminator for a double-stranded or partially

double-stranded DNA molecule. A padlock is a circular single stranded polynucleotide sequence tortionally linked to the polynucleotide molecule of this invention by hybridization between at least 10 consecutive nucleotides of the padlock and at least 10 consecutive nucleotides on the first strand. See Fig. 1C and 1D.

In this first strand of the DNA molecule, at least one of the above-described terminator sequences is preferably located 5' to the promoter. Desirably, this first strand terminator sequence is located between 1 to 50 nucleotides 5' of the promoter. In another desirable embodiment, this first strand terminator sequence is located between 20 to 40 nucleotides 5' of the promoter. In another desirable embodiment, this first strand terminator sequence is located between 10 to 30 nucleotides 5' of the promoter. In one embodiment, therefore, a padlock terminator (or any other suitable terminator or the complement of a ribonucleolytic or catalytic site) may be present within the 5' untranslated region of the first strand.

In still another embodiment, a first strand terminator sequence, independently selected from the above list, is located 3' to the polyA site, outside of the expression cassette. This first strand terminator sequence is desirably located at least 100 to 150 nucleotides 3' from the polyA site, or immediately following the end of the polyA sequence, of the expression cassette. Thus, in another embodiment, a padlock terminator (or any other suitable terminator or the complement of a ribonucleolytic or catalytic site) may be present within the 3' untranslated region of the first strand.

Further, in another embodiment, both the 5' and 3' terminator sequences are employed, flanking an expression cassette on the first strand.

In yet another embodiment of the first strand of the DNA molecule, in addition to the 5' and 3' terminator sequences that flank the expression cassette, additional optional terminator sequences are located on the first strand outside of the expression cassette sequence. In still another embodiment of this invention, at least one RNA instability sequence [see e.g., A. M. Curatola *et al*, Mol. Cell. Biol., 15(11):6331-6340 (Nov. 1995); A. M. Zubiaga *et al*, Mol. Cell. Biol., 15(4):2219-2230 (Apr. 1995)] is located on the first strand, preferably outside of the expression

cassette sequence. Preferably the complement of an RNA instability sequence is located 3' to the 3' flanking terminator sequence or 5' to the 5' flanking terminator sequence. Figs. 1A-1D illustrate schematic versions of several of the preferred embodiments.

In still another embodiment, a padlock terminator (or any other suitable terminator or the complement of a ribonucleolytic or catalytic site) is located within the expression cassette, and more particularly within the coding sequence of the selected polynucleotide sequence. For example, in Fig. 1D, the padlock terminator is located in the coding sequence of the polynucleotide sequence. When in this position, the terminator sequence does not impede transcription from the portion of the second strand complementary to the expression cassette, nor effect the biological function of the protein to be expressed. In other words, the presence of the terminator sequence in the first strand cannot prevent or impede the transcription from the second strand, so that the selected polynucleotide sequence is always correctly transcribed and expressed, retaining its biological function, regardless of the position of the first strand terminator sequence. In this position, the terminator sequence within the coding region may be any of the above-identified terminators, including a padlock probe described above, provided that it not impede transcription from the second strand or effect the biological function of the expressed protein.

Still as an alternative or additional embodiment, the first strand avoids any modification which results in the introduction of an ATG (start codon) or a Kozak region. Such modifications, alone or in combination, will ideally prevent unwanted transcription of antisense RNA. Where possible, however, added assurance against formation of ds RNA may be achieved by adding, at one or more sites within the coding strand, the complement of an RNA instability sequence. Any undesired antisense RNA inadvertently formed from this strand will contain an instability sequence promoting RNA degradation and will not be available to hybridize with sense RNA.

As one desirable modification, the first strand of the DNA molecule lacks any inverted complementary repeat sequences of greater than seven consecutive

"wobbled" codon prevents the transcript made from the DNA molecule in a host cell from undesirably or unintentionally shutting off or down-regulating a homologous polynucleotide sequence which is native to, or essential to, the transfected host cell. See Figs. 2A and 2B. By "substantially all" wobble bases is meant wobbling enough codons to destroy the homology between the selected polynucleotide and any polynucleotide in the host cell, so as to prevent the shutting off of the polynucleotide sequence in the host cell.

Where the DNA is produced recombinantly, the wobble bases may be altered by well-known mutational techniques. Where the DNA is synthesized, the wobble codon bases are deliberately made to avoid a match with native sequences in the intended host cell or organism. The wobble bases may be selected to incorporate preference codons to optimize codons for expression in a selected host cell, provided that the aberrant RNA structures discussed above are avoided. See, e.g., United States Patent No. 5,786,464, which teaches the selection of preferred codons.

As used herein and as known in the art, the terms "homology" or "homologous" refer to the degree of sequence relatedness between two polypeptide or two polynucleotide sequences as determined by the identity of the match between two lengths of such sequences. Both identity and homology can be readily calculated by methods extant in the prior art [See, e.g., COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A.M., ed., Oxford University Press, New York, (1988); BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed., Academic Press, New York, (1993); COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, (1994); SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heinje, G., Academic Press, (1987); and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, (1991)]. Methods commonly employed to determine identity or homology between two sequences include, but are not limited to, those disclosed in GUIDE TO HUGE COMPUTERS, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and H. Carillo and D. Lipton, SIAM J. Applied Math., 48:1073 (1988). Preferred methods to determine identity or homology are

designed to give the largest match between the two sequences tested. Preferred computer program methods to determine identity and homology between two sequences include, but are not limited to, the algorithm BESTFIT from the GCG program package [J. Devereux et al., Nucl. Acids Res., 12(1):387 (1984)], the related MACVECTOR program (Oxford), and the FASTA (Pearson) programs. The use of such computer programs enable the design of suitable DNA and RNA molecules desired for use in the invention. The algorithm and/or the degree of homology necessary for any particular DNA or RNA molecule may be selected by one of skill in the art. It should be understood that selection of the necessary homology, selection of the defaults for the program and selection of the program employed to calculate homology is within the skill of the art, given the teachings of this specification and the knowledge extant in the scientific literature.

B. The Second Strand

The double-stranded or partially double-stranded DNA molecule of this invention, or an alternative single-stranded DNA molecule of this invention, comprises preferably a second "transcription template" strand. While the second strand is ordinarily 100% complementary to the first strand in plasmids produced in bacteria, this is not essential, as, e.g., where at least one of the strands is produced synthetically and combined with a substantially homologous strand. Provided that there is sufficient homology between the two strands that the T_m is high enough to cause the strands to stay together under appropriate conditions, certain areas of non-homology with the first strand may be present on the second strand.

The second strand comprises at least one second strand terminator sequence which terminates any transcription initiated on the second strand. The second strand terminator sequence is located on the second strand in a sequence of the second strand which is not complementary to the expression cassette sequence. Preferably, the second strand of the DNA molecule contains more than one second strand terminator sequence. More preferably, the second strand contains from two terminator sequences to one terminator sequence for every 100 nucleotides of second strand outside of the sequence complementary to the expression cassette. Another

preferred embodiment contains one terminator sequence for every 500 nucleotides of second strand in the region which is not complementary to the expression cassette of the first strand. If more than one second strand terminator sequence is used, the multiple terminator sequences may be interspersed in the region of the second strand which is not complementary to the expression cassette, as described above.

In another embodiment, where it is desirable to include a terminator sequence at one or more positions on the first sense strand, including within the cistron or expression cassette, even within the gene or polynucleotide of interest, the second antisense DNA strand may have some degree of non-homology which needs to be transcribed into the mRNA. Still another embodiment is a second strand that contains one or more transcription terminators or RNA instability sequences outside of the region complementary to the coding region on the first strand. Changes in the second antisense strand can ordinarily be made only insofar as such changes will nevertheless yield a functional polypeptide and will not adversely effect transcription. While *cis* terminator or instability sequences may be encoded within the sense strand of a plasmid, nucleotides on the second, antisense or transcribed strand must include only conservative or nonsense mutations or other mutations which do not change or do not adversely affect the resulting amino acid sequence. In such embodiments, the function of any expressed protein must not be adversely affected.

Each terminator sequence on the second strand may be the same or each may each be a different terminator sequence. These terminator sequences include the sequences identified above as first strand terminator sequences. In one embodiment, a terminator sequence may be a bacterial terminator, such as the rrnB or trpA. In another embodiment, a terminator sequence may be a bacteriophage terminator, e.g., a lambda 5S RNA terminator or the bacteriophage P1 head protein terminator. Still another desirable terminator sequence which may be employed is a polynucleic acid sequence that provides a ribozyme cleavage site or a ribonucleic acid cleavage site, followed 3' by a eukaryotic pause site, e.g., a pause site for RNA pol II or an α-globin terminator. Further, a histone mRNA processing signal is a useful terminator sequence for the second strand. Similarly the terminator for the mammalian

gastrin gene, the gastrin terminator, is also useful. Both rho-dependent terminators and rho-independent terminators are also sequences which may be employed for this purpose in the second strand. Additionally, a padlock terminator sequence as described above is a useful terminator for the second strand if it is part of a double-stranded or partially double-stranded DNA molecule.

In one embodiment of a second strand of this invention, the terminator sequence is located in the region of the second strand which is not complementary to the expression cassette, and is positioned immediately 3' with reference to the first strand poly A site (see Fig. 1B). In another embodiment of this invention, a second strand terminator sequence is located less than 200 nucleotides from the poly A site in the region of the second strand which is not complementary to the expression cassette sequence (see Fig. 1C). An alternative embodiment of the second strand contains a terminator sequence is located 5' with reference to the first strand promoter sequence (see Figs. 1A-1C). A preferred embodiment contains multiple terminator sequences interspersed throughout the region of the second strand which is not complementary to the expression cassette of the first strand (see Figs. 1B and 1C).

Additional embodiments of the second strand of the DNA molecule involve the second strand additionally containing at least one RNA instability sequence located on the region of the second strand sequence which is not complementary to the expression cassette sequence. Preferably, more than a single instability sequence is present in that region of the second strand. Further, as with the first strand, the second strand preferably does not contain any complementary inverted repeat sequence of greater than seven nucleotides for use in mammalian cells or mammals or four nucleotides for use in invertebrate cells or organisms.

As another embodiment of a second strand of a DNA molecule of this invention, the "wobble" nucleotides in the second strand can be manipulated to alter the nucleotide sequence and prevent or eliminate any inverted complementary repeats in the sequence. This manner of eliminating such repeats permits the DNA to avoid being transcribed into RNA that will form double-stranded regions. Alternatively, an embodiment of the second strand of the DNA molecule of this invention, involves

altering the wobble nucleotides in substantially all of the codons in that portion of the second strand that is complementary to the selected polynucleotide sequence, where there is a possibility that the selected polynucleotide sequence in the molecule is too homologous with a polynucleotide sequence already present in the host cell. By altering only those bases which change the codon without changing the encoded amino acid, the selected polynucleotide sequence thus encodes the same amino acid sequence, but provides a nucleotide sequence which is substantially nonhomologous to a desired polynucleotide sequence in a host cell or host organism. This area of non-complementarity in the "wobbled" codon prevents the transcript made from the DNA molecule in a host cell from undesirably or unintentionally shutting off or down-regulating a homologous polynucleotide sequence which is native to, or essential to, the transfected host cell. See Figs. 2A and 2B. By "substantially all" wobble bases is meant wobbling enough codons to destroy the homology between the selected polynucleotide and any polynucleotide in the host cell, so as to prevent the shutting off of the polynucleotide sequence in the host cell. Where the DNA is produced recombinantly, the wobble bases may be altered by well-known mutational techniques. Where the DNA is synthesized, the wobble bases are deliberately made to avoid a match with native sequences in the intended host cell or organism.

The DNA molecules of this invention, whether double-stranded or partially double-stranded, and comprising a first and a second strand as described above, or a single strand, formed of either the first or the second strand as described above, permits the nucleotide sequence which is transcribed within the cell to become a single stranded RNA sense or anti-sense strand which lacks the capacity to form any significant double strandedness. The DNA molecule can provide a single stranded RNA sequence comprising both a sense polynucleotide sequence and an anti-sense polynucleotide sequence, optionally separated by a non-base paired polynucleotide sequence.

These DNA molecules of the invention may be prepared and used in the methods described in detail below.

II. RNA Molecules of the Invention

Another composition according to this invention is a substantially single-stranded RNA molecule, comprising a sequence of ribonucleic acids having a 5' end and a 3' end, which is designed to prevent the formation of stable double-stranded RNA or partially double-stranded RNA molecules in the host cell. Optionally, this molecule can contain a 5'ATG codon. Where the RNA is not intended to be translated, a 5' ATG is not required, e.g., for catalytic RNA molecules, such as ribozymes or antisense RNA. Optionally, regulatory regions such as Kozak sequences precede the 5' ATG codon. This single-stranded RNA molecule may be a linear molecule. Alternatively, this single-stranded RNA molecule may be circular.

This single stranded RNA molecule comprises a selected polynucleotide sequence having a selected biological function when translated in a host cell. The selected polynucleotide sequence may be a coding sequence, that is, it is translated to express a protein or a functional fragment thereof. Alternatively, the selected sequence may be non-coding, but may have a regulatory function or other biological function. The selected polynucleotide sequence with the biological function may be selected from the sequences described above for the first strand of the DNA molecule.

This single-stranded RNA polynucleotide sequence is between about 100 to 10,000 polynucleotides in length. At present the sequence is most desirably at least 200 polynucleotides in length, but it can range in one embodiment from 200 to 8000 polynucleotides in length. In another embodiment, the RNA molecule can be less than 7500 polynucleotides in length. In still another embodiment the RNA molecule can have a sequence length less than about 5000 polynucleotides. In yet another embodiment the RNA molecule can have a sequence length less than about 2000 polynucleotides. In still another embodiment the RNA molecule can have a sequence length less than about 1000 polynucleotides. In yet another embodiment the RNA molecule can have a sequence length less than about 750 polynucleotides.

In one embodiment, the single-stranded RNA molecule may possess a small hairpin sequence at the 3' end, i.e., a sequence of no more than 5 nucleotides of

sequence which is native to, or essential to, the transfected host cell. By "substantially all" wobble bases is meant wobbling enough codons to destroy the homology between the selected polynucleotide and any polynucleotide in the host cell, so as to prevent the shutting off of the polynucleotide sequence in the host cell. Where the RNA is produced recombinantly, the wobble bases may be altered by well-known mutational techniques. Where the RNA is synthesized, the wobble bases are deliberately made to avoid a match with native sequences in the intended host cell or organism.

In another embodiment, where the single stranded RNA molecule contains a chromosomal copy of a gene in the intended host cell, the single stranded RNA is prepared to be indistinguishable from the chromosomal RNA. The molecule contains no heterologous sequences flanking the selected polynucleotide sequence, which latter sequence is identical in ribonucleic acid sequence with the native sequence. This molecule is designed to avoid aberrently produced, partially double-stranded RNA with heterologous flanking sequences, which may cause the cell to shut off the chromosomal gene.

In still another embodiment of this invention, the single-stranded RNA molecule comprises a cap at the 5' end of the molecule. In another embodiment, the single-stranded RNA molecule has no cap at the 5' end of the sequence.

In still another embodiment of this invention, the single-stranded RNA molecule comprises a polyA sequence at the 3' end of the molecule. In another embodiment, the single-stranded RNA molecule has no polyA sequence at the 3' end of the sequence.

In still another embodiment, the single-stranded RNA molecule has attached at the 3' hydroxy group, a modification which functions as a chain terminator to block the extension of a hairpin (a double-stranded region). Among such chemical moieties include, without limitation, dideoxynucleotides (ddNTPs), 3' amino nucleotide triphosphates, 3' methyl nucleotide triphosphates, and 3' phosphorylthioate nucleotide triphosphates. Other chain terminators may be readily selected by those of skill in the art.

Still other embodiments of single-stranded RNA molecules of this invention include RNA sequences which provide a topological knot or lariat at the 3' end, which also functions to prevent chain extension. These structures can be prepared according to Smith and Nikonowicz, Biochem., 37:13486-13498 (1998).

Still other embodiments of single-stranded RNA molecules of this invention include RNA sequences which contain two or more of the above modifications. For example, an RNA molecule of this invention may have a 5' cap and a polyA tail; or no 5' cap, no inverted repeats, and a polyA tail. Still another embodiment has no inverted repeats, a chain terminator on the 3' end and no poly A tail. One of skill in the art may combine others of the modifications described above to prepare an RNA molecule of this invention.

It is desirable to avoid RNA molecules that are double stranded or partially double stranded to prevent the inadvertent shutting off or down-regulation of homologous polynucleotide sequences which are native to the host cell or otherwise essential to the host cell or organism. These RNA molecules of the invention may be prepared and used in the methods and compositions described in detail below.

III. Preparation of the DNA and RNA molecules

The DNA and RNA molecules described above may be designed and produced by use of known teachings of the art. Both of these molecules may be modified as described above to enhance expression of the selected sequence and prevent transcription of undesirable or aberrant polynucleotide species, thereby avoiding the unintended shutting off of polynucleotide sequences of the host cell or host organism.

These polynucleotide molecules may be designed by resort to conventional techniques such as those described in Sambrook, cited above or in Promega Protocols and Applications Guide, (3rd ed. 1996), eds. Doyle, ISBN No. 1-882274-57-1. For example, these molecules may be produced by enzymatic synthetic methods or chemical synthetic methods *in vitro*.

In one embodiment, an RNA molecule is made *in vitro* by conventional enzymatic synthetic methods using, for example, the bacteriophage T7, T3 or SP6

RNA polymerases according to the conventional methods described by such texts as cited above. For example, in one embodiment, an RNA or DNA molecule of this invention may be prepared in a host cell transfected with a plasmid containing the T7 promoter and another plasmid containing the T7 RNA polymerase.

In another embodiment, these molecules may be made by chemical synthetic methods *in vitro* [see, e.g., Q. Xu *et al*, Nucl. Acids Res., 24(18):3643-4 (Sept. 1996); N. Naryshkin *et al*, Bioorg. Khim., 22(9):691-8 (Sept. 1996); J. A. Grasby *et al*, Nucl. Acids Res., 21(19):4444-50 (Sept 1993); C. Chaix *et al*, Nucl. Acids Res., 17(18):7381-93 (1989); S.H. Chou *et al*, Biochem., 28(6):2422-35 (Mar. 1989); O. Odai *et al*, Nucl. Acids Symp. Ser., 21:105-6 (1989); N.A. Naryshkin *et al*, Bioorg. Khim, 22(9):691-8 (Sept. 1996); S. Sun *et al*, RNA, 3(11):1352-1363 (Nov. 1997); X. Zhang *et al*, Nucl. Acids Res., 25(20):3980-3 (Oct. 1997); S. M. Grvaznov *et al*, Nucl. Acids Res., 26 (18):4160-7 (Sept. 1998); M. Kadokura *et al*, Nucl. Acids Symp Ser, 37:77-8 (1997); A. Davison *et al*, Biomed. Pept. Proteins, Nucl. Acids, 2(1):1-6 (1996); and A. V. Mudrakovskaia *et al*, Bioorg. Khim., 17(6):819-22 (Jun. 1991)].

Alternatively, these molecules may be produced by recombinant means in a culture of host cells, and isolated therefrom. The DNA or RNA molecules useful in this invention can be made in a recombinant microorganism, e.g., bacteria and yeast or in a recombinant host cell, e.g., mammalian cells, and isolated from the cultures thereof by conventional techniques. See, e.g., the techniques described in Sambrook, cited above, which is exemplary of laboratory manuals that detail these techniques, and the techniques described in US Patent Nos. 5,824,538; 5,877,159 and 5,643,771, incorporated herein by reference.

The circular RNA molecule can be prepared according to the techniques described in S. Wang *et al*, Nucl. Acids Res., 22(12):2326-33 (June 1994); Y. Matsumoto *et al*, Proc. Natl. Acad. Sci., USA, 87(19)7628-32 (Oct. 1990); Proc. Natl. Acad. Sci., USA, 91(8):3117-21 (Apr. 1994); M. Tsagris *et al*, Nucl. Acids Res., 19(7):1605-12 (Apr. 1991); S. Braun *et al*, Nucl. Acids Res., 24(21):4152-7 (Nov. 1996); Z. Pasman *et al*, RNA, 2(6):603-10 (Jun. 1996); P. G. Zaphiropoulos, Proc.

Natl. Acad. Sci., USA, 93(13):6536-41 (Jun. 1996); D. Beaudry *et al*, Nucl. Acids Res., 23(15):3064-6 (Aug. 1995), all incorporated herein by reference.

The references above provide one of skill in the art with the techniques necessary to produce any of the following specific embodiments, given the teachings provided herein.

Such DNA and/or RNA molecules prepared or synthesized *in vitro* may be directly delivered to the host cell or to the host organism as polynucleotide molecules. Alternatively, desired DNA or RNA molecules may be provided to host cells in live, attenuated or killed, inactivated recombinant bacteria which are designed to contain the sequences necessary for the required DNA or RNA molecules of this invention. Such recombinant bacterial cells, fungal cells and the like can be prepared by using conventional techniques such as described in US Patent Nos. 5,824,538; 5,877,159 and 65,643,771, incorporated herein by reference. Microorganisms useful in preparing these delivery agents include those listed in the above cited reference, including, without limitation, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species of *Pseudomonas*, *Streptomyces*, *Staphylococcus* and *Shigella*.

The DNA or RNA molecules may be formed in the host cell or in the host by live, attenuated or killed, inactivated viruses, and particularly recombinant viruses carrying the required DNA or RNA polynucleotide sequence discussed above. Such viruses may be designed similarly to recombinant viruses presently used to deliver genes to cells for gene therapy and the like. Among useful viruses or viral sequences which may be manipulated to provide an RNA or DNA molecule to a host cell *in vivo* are, without limitation, alphavirus, adenovirus, adeno-associated virus, baculoviruses, delta virus, pox viruses, hepatitis viruses, herpes viruses, papova viruses (such as SV40), poliovirus, pseudorabies viruses, retroviruses, vaccinia viruses, positive and negative stranded RNA viruses, viroids, and virusoids, or portions thereof. These various viruses may be designed by applying conventional techniques such as described in M. Di Nocola *et al*, Cancer Gene Ther., 5(6):350-6 (1998), among others, with the teachings of the present invention.

Formation of the desired, above-described DNA or RNA molecules in the host cell may also occur in live, attenuated or killed, inactivated donor cells which have been transfected or infected *in vitro* with a synthetic RNA molecule or a DNA molecule or a recombinant virus as described above. These donor cells may then be administered to the host, as described in detail below. These donor cells are desirably cells of the host species as the host into which they are intended to be delivered, e.g., mammalian cells, such as C127, 3T3, CHO, HeLa, human kidney 293, BHK cell lines, and COS-7 cells, are useful host cells for mammals. Such donor cells can be made using techniques similar to those described in, e.g., Emerich *et al*, J. Neurosci., 16: 5168-81 (1996). Even more preferred, the donor cells may be harvested from the specific host to be treated and made into donor cells by *ex vivo* manipulation, akin to adoptive transfer techniques, such as those described in D. B. Kohn *et al*, Nature Med., 4(7):775-80 (1998).

Finally, the molecules of this invention can also be made into a mixture of synthetic RNA molecules or synthetic DNA delivery molecules as described above, or as or in recombinant bacteria, cells, and viruses. The composition of the mixture may be readily selected by one of skill in the art.

IV. Pharmaceutical (Therapeutic or Prophylactic), Diagnostic or Research Compositions and Methods of the Invention

The compositions of this invention are useful in an *in vitro* or tissue culture method for enhancing the efficiency of expression of a selected polynucleotide sequence in a host cell, or similarly useful if the same method (except for the recovery step) is employed to express the selected polynucleotide efficiently *in vivo* or *ex vivo*. One embodiment of the method comprises the step of transfecting the host cell with a double stranded or partially double stranded DNA molecule or an RNA molecule described above, thereby inhibiting the formation of aberrant polynucleotide species transcribed or translated from the polynucleotide molecule in the host cell. In an embodiment of this method where the host cell is in a test tube or tissue culture, this method enables the expression and recovery of maximal amounts of the product

encoded by the polynucleotide product from the host cell. For example, the host cell may be conventionally lysed and the product collected; or if the product is secreted, it may be collected from media by conventional techniques.

Where the host is a living mammal, the methods involve administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule as described above, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier to a mammalian subject. The compositions of this invention for pharmaceutical use desirably contain the DNA or RNA molecules, or mixtures thereof, in a pharmaceutically acceptable carrier, with additional optional components for pharmaceutical delivery. The specific formulation of the pharmaceutical composition depends upon the form of the active agent.

Suitable pharmaceutically acceptable carriers facilitate administration of the polynucleotide compositions of this invention, but are physiologically inert and/or nonharmful. Carriers may be selected by one of skill in the art. Such carriers include but are not limited to, sterile saline, phosphate buffered saline, dextrose, sterilized water, glycerol, ethanol, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, olive oil, sesame oil, and water and combinations thereof. Additionally, the carrier or diluent may include a time delay material, such as glycerol monostearate or glycerol distearate alone or with a wax. In addition, slow release polymer formulations can be used. The formulation should suit also the mode of administration. Selection of an appropriate carrier in accordance with the mode of administration is routinely performed by those skilled in the art.

Such molecules of the invention may be introduced into cells as polynucleotides, by well known techniques for introducing DNA into cells. The molecules, in the case of phage and viral vectors, may also be and preferably are introduced into cells as packaged or encapsidated DNA or RNA virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

formulation for efficacy in the target human or animal. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable stabilizing ingredients which may be used include, for example, casamino acids, sucrose, gelatin, phenol red, N-Z amine, monopotassium diphosphate, lactose, lactalbumin hydrolysate, and dried milk. A conventional adjuvant is used to attract leukocytes or enhance an immune response. Such adjuvants include, among others, Ribi, mineral oil and water, aluminum hydroxide, Amphigen, Avridine, L121/squalene, D-lactide-polylactide/glycoside, pluronic plyois, muramyl dipeptide, killed *Bordetella*, and saponins, such as Quil A.

In addition, other agents which may function as transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with the composition of this invention, include growth factors, cytokines and lymphokines such as alpha-interferon, gamma-interferon, platelet derived growth factor (PDGF), colony stimulating factors, such as G-CSF, GM-CSF, tumor necrosis factor (TNF), epidermal growth factor (EGF), and the interleukins, such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12. Further, fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicular complexes such as squalene and hyaluronic acid may also be administered in conjunction with the compositions of the invention.

The pharmaceutical compositions may also contain other additives suitable for the selected mode of administration of the composition. Thus, these compositions can contain additives suitable for administration via any conventional route of administration, including without limitation, parenteral administration, intraperitoneal administration, intravenous administration, intramuscular administration, subcutaneous administration, intradermal administration, oral administration, topical administration, intranasal administration, intra-pulmonary administration, rectal administration, vaginal administration, and the like. All such routes are suitable for administration of these

compositions, and may be selected depending on the agent used, patient and condition treated, and similar factors by an attending physician.

The composition of the invention may also involve lyophilized polynucleotides, which can be used with other pharmaceutically acceptable excipients for developing powder, liquid or suspension dosage forms, including those for intranasal or pulmonary applications. See, e.g., Remington: The Science and Practice of Pharmacy, Vol. 2, 19th edition (1995), e.g., Chapter 95 Aerosols; and International Patent Application No. PCT/US99/05547, the teachings of which are hereby incorporated by reference. Routes of administration for these compositions may be combined, if desired, or adjusted.

In some preferred embodiments, the pharmaceutical compositions of the invention are prepared for administration to mammalian subjects in the form of, for example, liquids, powders, aerosols, tablets, capsules, enteric coated tablets or capsules, or suppositories.

The compositions of the present invention, when used as pharmaceutical compositions, can comprise about 1 ng to about 20 mgs of polynucleotide molecules e.g., the synthetic RNA molecules or DNA molecules, plasmids, viral vectors, recombinant viruses, and mixtures thereof. In some preferred embodiments, the compositions contain about 10 ng to about 10 mgs of polynucleotide sequences. In other embodiments, the pharmaceutical compositions contain about 0.1 to about 500 μg polynucleotide sequences. In some preferred embodiments, the compositions contain about 1 to about 350 μg polynucleotide sequences. In still other preferred embodiments, the pharmaceutical compositions contain about 25 to about 250 μg of the polynucleotide sequences. In some preferred embodiments, the vaccines and therapeutics contain about 100 μg of the polynucleotide sequences.

The compositions of the present invention in which the DNA or RNA molecules are delivered in donor cells or bacterium can be delivered in dosages of between about 1 cell to about 10^7 cells/dose. Similarly, where the delivery agent is a live recombinant virus, a suitable vector-based composition contains between $1x10^2$ pfu to $1x10^{12}$ pfu per dose.

The above dosage ranges are guidelines only. In general, the pharmaceutical compositions are administered in an amount effective to treat or prevent the diseases, disorders or infections for which it is designed. The amount of the pharmaceutical composition in a dosage unit employed will be determined empirically, based on the response of cells *in vitro* and response of experimental animals to the compositions of this invention It will be appreciated that optimum dosage will be determined by standard methods for each treatment modality and indication. Thus the dose, timing, route of administration, and need for readministration of these compositions may be determined by one of skill in the art, taking into account the condition being treated, its severity, complicating conditions, and such factors as the age, and physical condition of the mammalian subject, the employment of other active compounds, and the like.

Still another embodiment of this invention is the use of the DNA and RNA molecules of this invention to prevent the unintentional or undesirable shutting off or down regulating of polynucleotide sequences which are essential or desirable in a host cell transfected with a polynucleotide molecule containing a polynucleotide sequence homologous to a polynucleotide sequence native to the host cell. This method comprises the steps of administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule as described above, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier.

Depending upon the identity of the selected polynucleotide sequence in the molecules described above, the compositions, pharmaceutical compositions, dosages and modes of administration described above are particularly desirable for the treatment of a variety of disorders that plague vertebrates, especially mammals, but also avians and other fowl, as well as invertebrates, such as fish, including infections by heterologous pathogenic organisms, either extracellular or intracellular pathogens. Additionally, the compositions of this invention are useful in preventing infection of a host with a pathogen, or in treating cancers. Further, these compositions are useful in the treatment of inherited or genetic disorders by expressing efficiently a polynucleotide protein or function which the host lacks.

One of skill in the art, given this disclosure can readily select viral families and genera, or pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites, for which therapeutic or prophylactic compositions according to the present invention can be made. See, e.g., the tables of such pathogens in general immunology texts and in U. S. Patent No. 5,593,972, incorporated by reference herein. One of skill in the art can readily select disorders described above, and can also readily design the appropriate selected polynucleotide sequences for use in treatment, or prophylaxis of a disease.

V. Other Methods of The Present Invention

The compositions described above, and the general methods of using these compositions to enhance the expression of a selected polynucleotide sequence in a host cell and thereby reduce the production or transcription or aberrant sequences can also be applied to a variety of research, and *in vitro* applications, where such efficient expression is essential. These compositions and methods may also be applied to manipulate plant cells and insect cells, and the hosts may similarly be plants and insects, among the other organisms mentioned previously.

Similarly, application of this method can be used to make cell lines of mammalian, bacterial, yeast, fungal, insect, plant, and other origins which efficiently produce a selected polynucleotide product. These molecules may also be employed to increase the efficiency of stable cell lines used to produce certain polynucleotide-encoded products. Such manipulated cells may be employed in producing recombinant proteins for pharmaceutical use in animals, for vaccine use in animals, as well as for a variety of uses in the agricultural fields. Such cells may also be employed in conventional testing assays for drugs or other useful compounds, or for drug screening and development assays, etc. Still other uses are expected to be obvious to the person of skill in the art given the teachings herein.

The following examples illustrate methods for preparing the compositions and using the compositions of this invention to increase the efficiency of transcription and thus expression of a selected polynucleotide sequence, and to reduce or inhibit

expression of aberrant (unintended) polynucleotide sequences. It is understood by one of skill in the art, that other selections for the various selected polynucleotides of the compositions, and RNA and/or DNA molecules may be readily made as taught by this specification. These examples are illustrative only and do not limit the scope of the invention.

EXAMPLE 1: A DNA Plasmid Containing Terminator Sequences on the Coding Strand using Mouse Interleukin 12 (mIL12) p40 as the Selected Polynucleotide

Experiments with expression of murine IL12 p40 from DNA plasmid constructs have been reported in literature. In the absence of an immune response (non-antigenic in mouse), the expression of the IL12 p40 protein in serum peaks at day 8, and decreases to basal (endogenous) levels by day 50.

In the following example, the plasmid sequence is modified by the insertion of terminator sequences to prevent the formation of aberrant RNA. The cloning sites in the mIL12 expression vector are shown in Fig. 6, as a HpaI site at nt#4700 (downstream of the polyA site), and an SphI site at nt#2568 (upstream of the CMV promoter/enhancer/ RSV enhancer module). The plasmid encoding mIL12 (Fig. 6) is modified to contain sequences (elements) that cause termination of elongation. The terminator sequences identified below are placed upstream to the human CMV promoter at the promoter cloning site, and at about 150 bases downstream of the SV40 polyadenylation sequence. When the terminator is used upstream of the promoter, a polyadenylation sequence is also cloned 150 base pairs upstream of the terminator sequence, since some termination sequences efficiently perform only when associated with a polyA site.

The terminator sequences inserted into the plasmids include the following: Bacterial polymerase terminators which are *rho* dependent and *rho* independent are *trp*A (Genbank accession #E02304) and *rrn*BT1T2 (Amersham-Pharmacia Biotech catalog #27-4925-01, Brosius, J., Gene, 27:151 (1984), Genbank accession #U13859), respectively. These sequences are isolated from existing vectors available from Pharmacia Biotech or these sequences are readily synthesized (Genbank accession

bacterial terminator, a bacteriophage terminator, a sequence comprising a ribozyme cleavage site or a ribonucleic acid cleavage site followed by a eucaryotic RNA pause site or an α-globin terminator, a histone processing signal, a rho-dependent terminator, and a rho-independent terminator.

35. A pharmaceutical composition comprising a polynucleotide molecule of any of claims 33-34, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier.

36. A substantially single-stranded RNA molecule comprising a 5' end, a ribonucleotide sequence having a selected biological function when translated in a host cell, and a 3' end, said molecule being incapable of stably forming a double-stranded or partially double-stranded RNA molecule.

37. The molecule according to claim 36 wherein wobble nucleotides are altered to prevent the occurrence of an inverted complementary repeat.

38. The molecule according to claim 36, modified to prevent the extension of a hairpin at the 3' end.

39. The molecule according to claim 36 wherein said molecule contains no inverted complementary repeat sequences of greater than 7 nucleotides in length.

40. The molecule according to claim 36 wherein said molecule contains no inverted complementary repeat sequences of greater than 4 nucleotides in length.

41. The molecule according to claim 36 which comprises a cap at the 5' end of said molecule.

42. The molecule according to claim 38 wherein said modification comprises attached a chain terminator at the 3' end of said sequence.

43. The molecule according to claim 36 which comprises a Kozak sequence positioned in said sequence 5' to the 5' codon.

44. The molecule according to claim 36 which comprises a polyA tail.

45. The molecule according to claim 36 which comprises no polyA tail.

46. A substantially single-stranded RNA molecule wherein wobble bases in substantially all codons in the portion of the molecule sequence that comprises a selected polynucleotide sequence are modified to encode the same amino acid sequence, but provide a nucleotide sequence which is substantially nonhomologous to a polynucleotide sequence present in a host cell.

47. A pharmaceutical composition comprising a polynucleotide molecule of any of claims 36-45, an optional agent that facilitates RNA uptake in a cell, and a suitable pharmaceutically acceptable carrier.

48. A method for enhancing the efficiency of expression of a selected polynucleotide sequence in a host cell, said method comprising the step of transfecting said host cell with a double stranded polynucleotide molecule comprising a first coding strand and a second transcription template strand,

(a) said first coding strand comprising

(i) an expression cassette sequence which comprises, from 5' to 3', a promoter, a selected polynucleotide sequence the expression of which is controlled by said promoter, and a polyadenylation site, and

(ii) at least one first strand terminator sequence; and

(b) said second strand complementary to said first strand, wherein the portions of said second strand sequence complementary to the first strand terminator sequence do not impede transcription from the second strand sequence complementary to said first strand expression cassette, and wherein said second strand comprises at least one second strand terminator sequence which terminates transcription initiated on said second strand outside of the second strand sequence that is complementary to said first strand expression cassette,

thereby inhibiting the formation of aberrant polynucleotide sequences transcribed from said polynucleotide molecule in said host cell.

49. The method according to claim 48, wherein said first strand terminator sequence is selected from the group consisting of a terminator sequence located 5' to said promoter, a terminator sequence located 3' to said polyadenylation site, a terminator sequence located on said first strand outside of said expression cassette sequence, and a terminator sequence located within the selected polynucleotide sequence of said expression cassette sequence.

50. The method according to claim 48, wherein said second strand terminator sequence is located on said second strand in a sequence which is not complementary to said first strand expression cassette sequence.

51. A method for treating a host subject comprising administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule of any of claims 1-31, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier.

52. A method for enhancing the efficiency of expression of a selected polynucleotide sequence in a host cell, said method comprising the step of transfecting said host cell with a substantially single-stranded RNA molecule comprising a 5' end, a ribonucleotide sequence having a selected biological function when translated in a host cell, and a 3' end, said molecule being incapable of stably forming a double-stranded or partially double-stranded RNA molecule.

53. A method for treating a host subject comprising administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule of any of claims 36 through 45, an optional agent that facilitates RNA uptake in a cell, and a suitable pharmaceutically acceptable carrier.

54. A method for preventing the inadvertent shutting off or down regulation of a polynucleotide sequence present in a host cell transfected with a polynucleotide molecule containing a polynucleotide sequence homologous to said polynucleotide sequence, said method comprising the steps of :

administering an effective amount of a pharmaceutical composition comprising a polynucleotide molecule of any of claims 1 through 31 and 36 through 45, an optional agent that facilitates polynucleotide uptake in a cell, and a suitable pharmaceutically acceptable carrier.

FIG. 1A

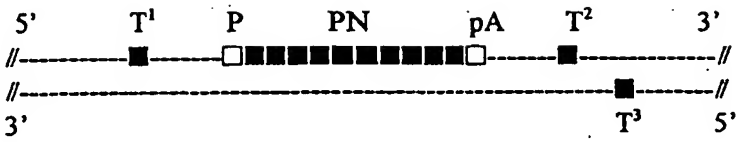

FIG. 1B

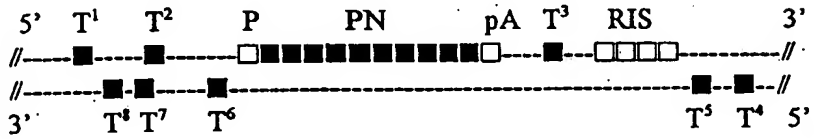

FIG. 1C

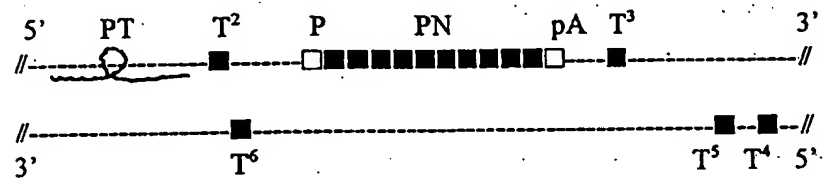

FIG. 1D